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A Reliable Method for Testing the Sensitivity of *Botryotinia fuckeliana* to Anilinopyrimidines *In Vitro*

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Abstract: Cyprodinil is a representative of the new class of broad-spectrum anilinopyrimidine fungicides. The effect of cyprodinil on mycelial growth of *Botryotinia fuckeliana* on solid agar medium depends on the composition of the medium and on the age of the mycelium to be used for the bioassay. An in-vitro method was developed to study the sensitivity distribution to cyprodinil in two wild-type populations of *B. fuckeliana* from fruits of strawberry with grey mould. To validate the applicability of the method, sensitivities to cyprodinil, mepanipyrim and pyrimethanil were monitored in populations of *B. fuckeliana* from grapes with grey mould from different vineyards, including one trial vineyard where reduced performance of cyprodinil had been encountered. The monitoring procedure was based on the inhibition of the mycelial growth on a synthetic medium containing L-asparagine (asp-agar) amended with the active ingredients. The mycelium was grown on asp-agar discs, starting from a spore suspension, for 17 h prior to the beginning of the test. This procedure proved to be efficient. The two wild-type populations from different sampling sites showed similar distributions of the sensitivity to cyprodinil. Some isolates from the trial site with reduced performance of anilinopyrimidines showed reduced sensitivities to cyprodinil, mepanipyrim and pyrimethanil, demonstrating cross-resistance between these anilinopyrimidines.

Key words: *Botryotinia fuckeliana*, cyprodinil, mepanipyrim, pyrimethanil, anilinopyrimidines, monitoring, sensitivity test, baseline

1 INTRODUCTION

The anilinopyrimidines cyprodinil [CGA 219417; *N*-(4-cyclopropyl-6-methylpyrimidin-2-yl)aniline], mepanipyrim [*N*-(4-methyl-6-prop-1-ynylpyrimidin-2-yl)aniline] and pyrimethanil [*N*-(4,6-dimethylpyrimidin-2-yl) aniline] represent a new group of fungicides with a broad spectrum of activity.[1–4] The spectrum of fungal pathogens controlled by the anilinopyrimidines includes *Botryotinia fuckeliana* (de Bary) Whetzel, the causal agent of grey mould on a wide host range.[1–4] All three active ingredients were registered in 1995 in Switzerland for the first time.[5] The mode of action of the anilinopyrimidines is not fully understood. While inhibition of the biosynthesis of methionine was suggested as the site-specific mode of action of cyprodinil in *B. fuckeliana*, *Pseudocercosporella herpotrichoides* (Fron) Deighton and *Helminthosporium oryzae* B. de Haan,[6] it was also suggested that mepanipyrim and pyrimethanil repress the secretion of extracellular enzymes[7–9] that are involved in pathogenesis of *B. fuckeliana*.[1]

The activity of anilinopyrimidines in sensitivity tests such as the inhibition of mycelial growth or germination is low when complex media are used e.g. mepanipyrim hardly inhibited spore germination of *B. fuckeliana* at 100 $\mu g\ ml^{-1}$ and did not fully inhibit the mycelial growth at 1000 $\mu g\ ml^{-1}$ in laboratory tests.[7] The lack of a reliable test procedure posed a major obstacle for the monitoring of fungal population responses.

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Consequently, assessments of resistance risks and baseline sensitivities have not been provided for the anilinopyrimidines.

The present study was initiated to determine the range and frequency of sensitivities of two wild-type populations of *B. fuckeliana* to cyprodinil as a representative of the anilinopyrimidines. The monitoring procedure was used to study cross-resistance between anilinopyrimidines in a trial site with reduced performance of anilinopyrimidines. The aim of the study was to develop a simple, quick and inexpensive sensitivity test as the first step of resistance risk assessment.[10–12]

2 MATERIALS AND METHODS

2.1 Chemicals

The technical grade cyprodinil (Ciba-Geigy, Switzerland), pyrimethanil (AgrEvo, Germany) and mepanipyrim (Kumiai, Japan) (Fig. 1) were provided by the manufacturers. The other chemicals used were purchased from E. Merck, Switzerland.

2.2 Media

A complex medium (malt-agar: malt extract 15 g litre^{-1}, agar 15 g litre^{-1} in distilled water) and a synthetic medium containing L-asparagine (asp-agar), a modification of the medium described by Hammer *et al.*,[13] were used for mycelial growth tests. The asp-agar was prepared as follows: 1 g K_2HPO_4 and 1 g $MgSO_4 \cdot 7\ H_2O$ were each dissolved in 30 ml water (stocks I and II), 0·5 g KCl and 0·01 g $FeSO_4 \cdot 7\ H_2O$ were dissolved in 40 ml distilled water (stock III), 2 g L-asparagine and 15 g agar were dissolved in 400 ml water (stock IV) and 22 g glucose $\cdot$ 1 H_2O were dissolved in 490 ml water (stock V). Stocks I and II were pooled. The precipitate that was formed was dissolved by adding 10 M hydrochloric acid dropwise, then stock III was added. Again, the formation of a precipitate was observed, which dissolved after adding stock IV and autoclaving at 1·013 bar for 20 min. Stock V was autoclaved separately and then pooled with the rest. The pH values of all components ranged between 6·5 and 7·0. Cyprodinil, pyrimethanil or mepanipyrim, respectively, were dissolved in acetone before mixing with the agar that was cooled to 50°C. In all cases (including unamended control plates), the final amount of acetone added was 10 ml litre^{-1}. The agar was poured (20 ml each) into plastic Petri dishes with a diameter of 9 cm.

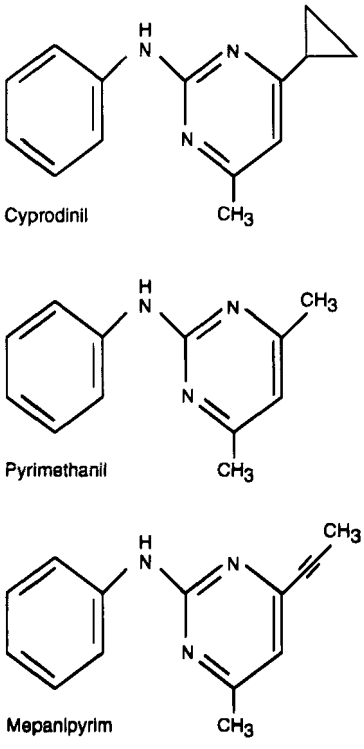

Fig. 1. Chemical structures of anilinopyrimidines.

2.3 Isolates

The isolates of *B. fuckeliana* used in this study are described in Table 1.

Sixty-two isolates of *B. fuckeliana* were provided by Ciba, Basle, Switzerland and 103 by the Swiss Federal Research Station, Wädenswil, Switzerland, culture collection.

Fifty infected strawberry fruits with distinct sporulating grey mould lesions were collected in June 1993 from 50 well-separated, unsprayed strawberry plants at two different sampling sites in north-eastern Switzerland. The isolation method has been described in detail elsewhere.[10,12]

A total of 60 infected grape berries with distinct sporulating grey mould lesions were collected in September 1994 in a test site of Ciba at Rūdlingen, Switzerland, where reduced performance of cyprodinil had been observed during 1994.[14] Experimental design and performance data from the trials at this site are described in detail elsewhere.[14] Twenty isolates (R 1–20) were collected from untreated grapes and 20 each were sampled from grapes that had been treated four times either with fludioxonil (R 21–40) a chemically unrelated compound, or with cyprodinil (R 41–60).

2.4 Sensitivity test

2.4.1 Influence of the media on the in-vitro activity

The sensitivities of ascospore isolate S and field isolate R were determined as follows. Three agar discs (8 mm diameter) were cut from malt-agar and asp-agar, respectively. The agar discs were inoculated with a conidial suspension (10^5 conidia ml^{-1}; 20 µl) and incubated for

TABLE 1
List of *Botryotinia fuckeliana* Isolates

Isolate	*Isolation year*	*Location*	*Host plant*
R	1988	Ascospore isolate (parental isolates from Oedischwend, Wädenswil, Switzerland, from untreated plot)	Strawberry
S	1991	France (from untreated plot)	Grape
CH 9·83	1983	Switzerland (ref. isolate Ciba)	Unknown
St 93 K1	1993	Stäfa, Switzerland (from untreated plot)	Grape
St 93 2	1993	Stäfa, Switzerland (from untreated plot)	Grape
K 1–50	1993	Knonau, Switzerland (from untreated plot)	Strawberry
M 1–50	1993	Mettmenstetten, Switzerland (from untreated plot)	Strawberry
R 1–20	1994	Rüdlingen, Switzerland (from untreated plot)	Grape
R 21–40	1994	Rüdlingen, Switzerland (four treatments with fludioxonil)	Grape
R 41–60	1994	Rüdlingen, Switzerland (four treatments with cyprodinil)	Grape

17 or 41 h at 20°C in the dark. Conidial germination was homogeneous; germination rates were $\geq 90\%$ in all experiments and no sporulation occurred. The agar discs with the young, homogeneous mycelium were then placed upside-down onto plates containing 20 ml of malt-agar or asp-agar with either no cyprodinil, or with cyprodinil at concentrations of 1 or 10 $\mu g\ ml^{-1}$. Plates were incubated for three days at 20°C. The mean colony diameter, minus the diameter of the inoculation disc, was measured and expressed as the percentage of the mean colony diameter of the untreated control. All experiments were repeated twice.

2.4.2 Influence of the age of the inoculum on the in-vitro activity
The sensitivity of ascospore isolate S was tested as described above; the agar discs used for inoculum, however, had been incubated for 17, 19, 21, 23, 25, 27 or 29 h prior to the transfer onto the plates containing asp-agar or malt-agar with either no fungicide, or cyprodinil at concentrations of 0·01, 0·03, 0·1, 0·3, 1, 3 or 10 $\mu g\ ml^{-1}$.

2.4.3 Cross-sensitivity/resistance of Botryotinia fuckeliana *to cyprodinil, mepanipyrim and pyrimethanil*
The sensitivities of isolates CH 9.83, St 93 Kl and St 93 2 were tested on asp-agar amended with cyprodinil, mepanipyrim or pyrimethanil each at concentrations of 0, 0·001, 0·01, 0·1, 1 or 10 $\mu g\ ml^{-1}$ using mycelium that had been incubated 17 h prior to the test as described above.

Mycelial growth of isolates R 1–60 was tested as described above on agar containing 0·03 $\mu g\ ml^{-1}$ cyprodinil, 0·1 $\mu g\ ml^{-1}$ pyrimethanil or 0·1 $\mu g\ ml^{-1}$ mepanipyrim. Isolates which were inhibited less than 50% (compared to the control on non-amended agar) were considered resistant.

2.4.4 Baselines
The cyprodinil sensitivity of 50 isolates (K 1–50, M 1–50) per strawberry field was determined at concentrations of 0, 0·001, 0·01, 0·03, 0·1, 1 or 10 $\mu g\ ml^{-1}$ as described above. EC_{50} values were determined after an incubation period of three days by regressing the relative growth (colony diameter on cyprodinil-amended agar divided by the diameter on unamended agar × 100) against the log of the fungicide concentration.

3 RESULTS

3.1 Influence of the media and the age of the inoculum on the in-vitro activity

The effect of anilinopyrimidine fungicides on *B. fuckeliana* was tested on malt-agar and on asp-agar with mycelium cultivated for different periods of time prior to the sensitivity test. Quantification of sensitivities was based on the inhibition of mycelial growth. The sensitivity to cyprodinil varied with regard to the composition of the medium and the age of the mycelium used as

TABLE 2
Mycelial Growth Inhibition of Two *Botryotinia fuckeliana* Isolates by Cyprodinil on Asp-agar and Malt-agar Inoculated with 17 and 41-h-old Mycelium

		Inhibition of mycelial growth (%)			
		Asp-agar		*Malt-agar*	
		Cyprodinil ($\mu g\ ml^{-1}$)			
Isolate	*Age of inoculum (h)*	*1*	*10*	*1*	*10*
R	17	30	58	18	37
S	17	100	100	100	100
R	41	19	40	2	30
S	41	91	100	11	44

inoculum (Table 2). Isolate R was less inhibited than isolate S for all variations of test media and time of mycelium incubation prior to the test. On malt-agar, isolate S was less inhibited if the mycelium had been incubated for 41 h instead of 17 h prior to the test; on asp-agar the inhibition of isolate S was more than 90%, independent of the length of the incubation period of the mycelium.

The influence of the test medium and the age of the mycelium used for the test on the in-vitro activity of cyprodinil was studied in more detail using isolate S (Table 3). The length of the incubation period of the inoculum did not influence cyprodinil sensitivity on asp-agar. On malt-agar, however, the EC_{50} value shifted from 0·01 $\mu g\ ml^{-1}$ when testing mycelium 17 h old to 1 $\mu g\ ml^{-1}$ when testing mycelium 23 h old.

3.2 Cross-sensitivity/resistance of *Botryotinia fuckeliana* to cyprodinil, mepanipyrim and pyrimethanil

As a standard method, the growth test starting from mycelium 17 h old on asp-agar was used to study cross-sensitivity to cyprodinil, mepanipyrim and pyrimethanil of three selected isolates. Isolate St 93 2 showed a decreased sensitivity to all three anilinopyrimidines in comparison with CH 9.83 and St 93 K1. Against isolate CH 9.83 and St 93 K1, cyprodinil was slightly more effective than mepanipyrim and pyrimethanil. EC_{50} values of St 93 2 were $>10\ \mu g\ ml^{-1}$ for all three active ingredients (Table 4).

3.3 Baselines

The variability in sensitivity to cyprodinil of two wild-type populations represented by 50 isolates of *B. fuckeliana* collected from strawberries never treated with fungicides was low (Table 5). No differences were apparent with regard to the cyprodinil sensitivity distributions of populations of *B. fuckeliana* between the two sampling sites (*t*-test, $P = 0{\cdot}06$). With regard to the inhibition of mycelial growth, dose–response curves of cyprodinil were characterized by a steep slope in a narrow range of concentrations with an inhibition >90% at 0·03 $\mu g\ ml^{-1}$ (Table 4). Variability of inhibi-

TABLE 3
Inhibition of Mycelial Growth of *Botryotinia fuckeliana* (Isolate S) by Cyprodinil Relative to the Age of the Mycelium Used as Inoculum

	Inhibition (%)													
	Asp-agar							*Malt-agar*						
Cyprodinil concentration ($\mu g\ ml^{-1}$)	*Age of inoculum (h)*													
	17	*19*	*21*	*23*	*25*	*27*	*29*	*17*	*19*	*21*	*23*	*25*	*27*	*29*
0·01	21	17	18	18	13	19	9	1	0	2	1	2	8	5
0·03	100	100	100	100	100	100	100	95	75	63	34	37	10	12
0·1	100	100	100	100	100	100	100	100	91	66	36	37	10	20
0·3	100	100	100	100	100	100	100	100	91	74	46	45	38	29
1	100	100	100	100	100	100	100	100	95	74	49	42	42	66
10	100	100	100	100	100	100	100	100	100	88	86	86	85	88

TABLE 4
Inhibition of Three Isolates of *Botryotinia fuckeliana* by Different Anilinopyrimidine Fungicides on Asp-agar Using 17-h-old Mycelium as Inoculum

Concentration ($\mu g\ ml^{-1}$)	*Inhibition of mycelium growth* (%)								
	Cyprodinil			*Mepanipyrim*			*Pyrimethanil*		
	CH 9·83	*St 93 K1*	*St 93 2*	*CH 9.83*	*St 93 K1*	*St 93 2*	*CH 9·83*	*St 93 K1*	*St 93 2*
0·001	0	2	1	1	10	0	0	0	0
0·01	85	86	5	2	11	3	4	11	2
0·1	100	100	4	97	97	2	97	97	2
1	100	100	4	100	100	2	100	100	4
10	100	100	36	100	100	21	100	100	15

tion of mycelial growth was greatest on asp-agar supplemented with 0·01 $\mu g\ ml^{-1}$ cyprodinil (Table 5).

3.4 Resistance monitoring

The trial site at Rüdlingen, Switzerland, where reduced performance of anilinopyrimidines had been observed in 1994[14] was monitored. From the plot which was treated four times with cyprodinil in 1994, isolates R 1–20 were inhibited less than 50% compared to the control when tested on the respective discriminatory doses of cyprodinil, mepanipyrim and pyrimethanil. In two neighbouring plots 20 samples each (R 21–60) were tested. In the unsprayed plot two isolates showed a reduced sensitivity to all three anilinopyrimidines and

TABLE 5
Sensitivity Distribution of 50 Isolates each of *Botryotinia fuckeliana* to Cyprodinil in a Mycelial Growth Test

Sampling site	EC_{50} ($\mu g\ ml^{-1}$)		*Inhibition at 0·01* $\mu g\ ml^{-1}$ (%)	
	Range	*Mean*[a]	*Range*	*Mean*[b]
Strawberry field 1	0·005–0·014	0·0075	32·4–98·9	74·8
Strawberry field 2	0·005–0·009	0·0068	53·1–100	87·2

[a] Mean of EC_{50} values of 50 isolates.
[b] Mean of inhibition of mycelial growth of 50 isolates on asp-agar supplemented with 0·1 $\mu g\ ml^{-1}$ cyprodinil.

TABLE 6
Resistance Monitoring: *Botryotinia fuckeliana* to Anilinopyrimidines, Rüdlingen, Switzerland, 1994

Treatment	*Number of isolates*	*Number of resistant isolates*		
		Cyprodinil[a]	*Mepanipyrim*[a]	*Pyrimethanil*[a]
Untreated	20	2[b]	2	2
Fludioxonil four treatments	20	3[b]	3	3
Cyprodinil four treatments	20	20	20	20
Reference isolate 1	CH 9·83	0	0	0
Reference isolate 2	St 93·2	1	1	1

[a] Discriminatory doses: cyprodinil = 0·03 $\mu g\ ml^{-1}$; mepanipyrim = 0·1 $\mu g\ ml^{-1}$; pyrimethanil = 0·1 $\mu g\ ml^{-1}$.
[b] The same five isolates were resistant to the three fungicides.

in the plot which was sprayed with fludioxonil four times in 1994 three isolates showed a reduced sensitivity to the anilinopyrimidines (Table 6).

4 DISCUSSION

Little or no effect of anilinopyrimidines has been observed on mycelial growth and germination of *B. fuckeliana* using complex media.[3,7]

Our investigations confirmed that complex media such as malt-agar were not appropriate for in-vitro testing of cyprodinil if mycelial plugs were used as inoculum having been cut from a colony grown on an agar plate for more than 23 h. This however, is the most common way to produce inoculum for testing mycelial growth.[15] Consequently, an adapted, inexpensive and rapid sensitivity test based on measurement of inhibition of mycelial growth on fungicide-amended synthetic agar medium was developed.

The in-vitro activity of cyprodinil depended on the medium used for the test. The incubation period of the inoculum did not affect the in-vitro activity if asp-agar was used. However, if malt-agar was used, the in-vitro activity correlated negatively with increasing age of the mycelium used for the bioassay. This explains the reported lack of in-vitro activity when complex media such as malt extract agar are used and when the mycelium to be tested has been cut from the margin of a colony that has been grown for several days.

Anilinopyrimidines hardly impair the germination process of B. fuckeliana.[7] The most sensitive step in the development of *B. fuckeliana* is the extension of the germ tube.[4,9] With the conversion of germ tubes into mycelium, the sensitivity of *B. fuckeliana* decreases. It must be assumed that the early phase of germ-tube elongation is the most critical phase because the energy reserves which are stored in the spore are used up. After the formation of a straight germ tube, intensive branching occurs, leading to a fungal colony. This colony is physiologically most active at its margin, while the colony centre may be used for storage. Malt-agar is a rich nutrient source compared to asp-agar. Germ-tube elongation is increased and the transition into mycelium occurs faster than on asp-agar. This might be the explanation for the observation that the effect of cyprodinil on mycelial growth of *B. fuckeliana* on solid agar medium depends on the composition of the medium and on the age of the mycelium to be used for the bioassay. In nature, however, it is more likely that *B. fuckeliana* will encounter a nutrient base of the asp-agar type rather than the rich malt-agar type. The early phase of germ-tube formation which is most sensitive to anilinopyrimidines is then prolonged.

B. fuckeliana was isolated from strawberries that had never been treated with fungicides. Two locations were chosen in north-eastern Switzerland at a distance of several kilometres from any crops treated with fungicides. All 50 wild-type isolates from each location were sensitive to cyprodinil. In a field trial of Ciba-Geigy in Rüdlingen, Switzerland, where anilinopyrimidines had been applied alone or in mixture since 1986, with up to four treatments per year, a loss of field efficacy occurred.[14] In the plot where cyprodinil was applied four times in 1994, all 20 isolates tested showed a reduced sensitivity to cyprodinil and were cross-resistant to mepanipyrim and pyrimethanil. Cross-resistance is, however, not unexpected, as the chemical structures of the three anilinopyrimidines are closely related, with differences restricted to the side chain at C6 (Fig. 1). In neighbouring plots the resistance frequency was 10 to 15%. However, due to the small distance between the plots[14] it cannot be excluded that resistant isolates were distributed in the vineyard by wind and rain splashes. The mycelial growth test on asp-agar is a valuable sensitivity test for monitoring the build-up of resistance. It is inexpensive, easy to perform and reliable. The test described in this paper is now used in other laboratories.[14,16,17] It can be used as a tool to evaluate antiresistance strategies[14] as well as contributing to a better understanding of the occurrence and the spread of resistant isolates.

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